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The biological activities of OM can be mediated by two different heterodimeric receptor complexes, the LIF/OM shared receptor and the OM-specific receptor which is composed of gp130 and a newly identified signal transducing subunit. In this study, we have investigated the growth inhibitory mechanism of OM on breast cancer cells and the receptor subtype involved in OM action. Our data show that the growth inhibitory activity of OM is partially due to its functional antagonism of breast cancer mitogens including EGF, EGF-like growth factor, and bFGF. The proliferative activities of these growth factors were totally abolished by cotreatment of breast cancer cells with OM. EGF binding and the induction of EGF-receptor tyrosine phosphorylation were not affected by OM, suggesting that OM interferes with the mitogenic signal at steps distal to the EGF receptor. Examination of protooncogene expression demonstrated that OM down regulates the *c-myc* gene in growth-inhibited breast cancer cells. The biological effects reported herein are not shared by the OM-related cytokines IL-6, IL-11, or LIF as demonstrated by the inability of these proteins to inhibit growth or modulate *c-myc* gene expression in breast cancer cells. Furthermore, the [^{125}I]OM binding to breast cancer cells can not be replaced by LIF suggesting a direct binding to OM specific receptor. Together these data suggest that the growth inhibitory activity is mediated predominantly through the OM-specific receptor, and activation of this receptor abrogates growth factors stimulation and down regulates the *c-myc* protooncogene.

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Jingwen Lin 4/29/96

PI - Signature Date

Table of Contents

Introduction

Human oncostatin M (OM), a product of activated T cells and macrophages, is a 28 kDa glycoprotein that regulates cell growth and differentiation. OM stimulates the growth of normal fibroblasts, normal vascular smooth muscle cells, and myeloma cells. OM also has been shown to inhibit the proliferation of a number of cell lines derived from human tumors including melanoma and lung carcinoma (1-7). The growth inhibitory or growth stimulatory activity exhibited by OM seems to depend on target cell type.

OM signal transduction occurs through two types of heterodimeric receptors (8-10). A shared receptor mediates both OM, LIF and CT-1 signals and is composed of the LIF receptor α subunit and gp130 (9,11). Many overlapping functions of OM and LIF are mediated through this common receptor. However, we and others have demonstrated that a specific OM receptor exists in certain cell types including breast cancer cells. (9,10,12). Therefore, in the grant, we originally proposed to isolate and characterize this OM specific receptor and we predicted that the OM-specific receptor transduces exclusively OM signals and would consist of gp130 as a binding subunit and a second subunit as an affinity conversion subunit. However, during our initial work to construct a cDNA library and to clone this second subunit, an abstract reporting the isolation of signaling subunit of OM receptor was published in the fall of 1994 at the International Cytokine Society Meeting. Bruce Mosley at Immunex reported the cloning of this subunit (13). Therefore, we decided not to waste our resource to continue to clone this subunit, instead to start a collaboration with Immunex to characterize the expression of this newly identified molecule in breast cancer and in normal mammary epithelial cells, and to investigate the functional roles of OM-specific receptor versus the OM/LIF shared receptor in OM mediated growth inhibition, since currently little is known about how the cellular growth response to OM is controlled at the receptor level, and the molecular mechanism(s) by which OM regulates cell growth remains largely uncharacterized.

In this report, we show that OM inhibits cellular proliferation of a large number of breast cancer cell lines that were developed from solid tumors and malignant effusions, and that growth inhibitory activity is mediated by the OM-specific receptor. Further, we demonstrate that OM antagonizes the mitogenic effects of several peptide growth factors that are required for the growth of breast cancer cells. Our data show that the proliferative activities of EGF, other members of the EGF family, and bFGF were inhibited by OM in a concentration-dependent manner under both anchorage-dependent and anchorage-independent conditions. Finally, examination of protooncogene expression indicates that the level of *c-myc* mRNA is greatly decreased in OM-treated breast cancer cells. This suggests that the mechanism(s) of growth inhibition by OM may involve down regulation of *c-myc*. In addition to breast cancer cells, we also examined the effect of OM and the expression of OM specific receptor in normal human mammary epithelial cells (HME). Our data show that OM plays an inhibitory and differentory role in HME cells. OM-specific receptor is highly expressed in HME cells, and the expression level of this receptor is diminished or totally lost in breast cancer cells. These data together suggest that OM may play a physiological role as a negative growth regulator. The deregulated expression of OM-specific receptor would result in abnormal growth of mammary epithelial cells. That may be one of the factors that contributes to breast malignancy.

Methods

Cell Proliferation Assay. Cells were seeded in 96-well tissue culture plates (Costar, Cambridge, MA) in IMDM medium containing 2 to 10% FBS at a density of 3000 cells/well in 100 μl of medium. Three to 5 hours after seeding, 50 μl of the same culture media containing various factors was added to each well. Three days later [^{3}H]thymidine (0.1 μCi/50 μl/well) in medium was added to the culture plates 4 hours prior to harvest. The amount of [^{3}H]thymidine incorporated into cells was measured using a liquid scintillation counter (Pharmacia, Piscataway, NJ). The differences in counts/min incorporated between experimental and control cultures were used as an index for DNA synthesis. Each data point represents the average of triplicate cultures and each experiment was performed at least 5 times.

Cell Number Counts. Cells ($1x10^5$) were seeded in 6-well tissue culture plates in IMDM (2.5 ml/well) containing 10% FBS. Three hours after seeding various factors, diluted in the same medium, were added to the cultures. After 3 days the cells were trypsinized and trypan blue was added (Sigma Chemical Co., St. Louis, MO). Cells excluding dye were considered to be viable cells and counted. Each experiment was performed at least 3 times.

Soft Agar Colony Assay. A basal layer of 0.5% agar (0.38 ml/well) (Difco Laboratories, Detriot, MI) in 10% FBS IMDM was added to 24 well culture plates as described. A 0.3% agar (0.35 ml/well) containing 10% FBS IMDM, 1.5×10^4 cells, and test factors were overlaid on the basal layer of agar. The plates were incubated at 37° C, 5% CO_2 for 5 to 7 days. Colonies with 20 cells or more were counted by light microscopy.

Iodination and Receptor Binding Assay. Purified human recombinant OM and EGF were radiolabeled by the chloramine T method to a specific activity of 1760 Ci/mmol for OM and 400 Ci/mmol for EGF. The binding assays were performed in 48 well tissue culture plates at a density of $1x10^5$cells/well in 150 μl of RPMI (supplemented with 20 mM HEPES, [pH 7.4], 0.01% NaN_3, and 1% bovine serum albumin) containing varying concentrations of radioligand in the absence or presence of a 100-fold excess of unlabeled ligand. Scatchard analysis of the binding data was conducted using Ligand, version 4.

Immunoprecipitation and Immunoblotting. H3922 cells were cultured in 60 mm culture plates for 2 days in 2% FBS IMDM with or without OM (100 ng/ml). The cells were then stimulated with 10 ng/ml of EGF for 10 minutes, or 100 ng/ml of OM for 15 minutes. Cells were rinsed with cold PBS and lysed with 0.5 ml of lysis buffer (50 mM Tris [pH 7.4], 1% NP-40, 0.25% sodium deoxycholate, 150 mM NaCl, 1 mM EGTA, 1 mM phenylmethylsulfonyl fluoride (PMSF), 1 mM Na_3VO_4, 1 mM NaF, 5 μg/ml of aprotinin, 1 μg/ml of leupeptin, and 1.25 μg/ml of pepstatin). The cell lysate (250 μl, approximately 1μg/μl of protein) was precleared with 50 μl of protein A-sepharose slurry and then incubated overnight with 30 μl of sepharose-conjugated anti-phosphotyrosine monoclonal antibody 4G10. The sepharose beads were collected by microcentrifugation and resuspended in 50 μl of 2X SDS sample buffer containing 5% β-mercaptoethanol. Immunoprecipitates were analyzed by western blot with anti-phosphotyrosine monoclonal antibody 4G10 using an enhanced chemiluminescence (ECL) detection system.

Northern Blot Analysis. Total cellular RNA was isolated by the method of Peppel and Baglioni. Approximately 25 μg of each total RNA sample was separated on a 1.0% formaldehyde agarose gel. RNA was capillary transferred to a Hybond N membrane before crosslinking to the membrane. Prehybridization and hybridization steps were performed under the conditions previously described. The blot was hybridized at 67° C to a 1.6 Kb ^{32}P-labeled human *c-myc* probe. The probe was labeled using 40 μCi [α-^{32}P] dCTP with random primed DNA labeling kit (Boehringer Mannheim Corp, Indianapolis, IN). The membrane was then washed once at ambient temperature with 2X SSC, 0.1% SDS and twice at 65° C with 0.1X SSC, 0.1% SDS. The membrane was then dried and exposed to X-OMAT scientific imaging film (Kodak, Rochester, NY) for 24 h at -80° C. The *c-jun* and *GAPDH* probes were prepared by random-primer labeling as described for the *c-myc* probe. All other steps in analysis of the membrane were also followed as described for the *c-myc* probe.

HME cell DNA synthesis measured by TCA precipitation. Cells were seeded in 24-well tissue culture plates (Costar, Cambridge, MA) in MEGM at a density of 1.2 x 10^4 cells/well in 400 μl of medium. Three to 5 hours after seeding, 50 μl of the same culture media containing various factors was added to each well. Three days later [^{3}H]thymidine (0.5 μCi/50 μl/well) in medium was added to the culture plates 4 hours prior to harvest. The cells were washed 2X with cold PBS, then 200 μl of 5% TCA was added to each well at 4°C and incubated for 20 min. After rinsing the cells with 3X PBS, 200 μl of 0.02% SDS was added to each well, and the amount of [^{3}H]thymidine incorporated into cells was measured using a liquid scintillation counter.

Results

Effect of OM on breast cancer cell morphology. After a general screening of the responses of the 10 breast cancer cell lines to OM, further studies to investigate the mechanisms of OM inhibitory activity were carried out in H3922 cell line. This cell line was derived from an infiltrating ductal tumor. These cells expressed the highest level of OM binding sites compared with the other breast cancer cell lines. In addition, H3922 cells responded to OM with strong growth regression and striking morphological changes. As illustrated in Figure 1, the majority of OM-treated cells exhibited cytoplasmic vesicles after 3 days of culture. These vesicles were rarely present in control cells. The OM effect on cell morphology was reversible, as withdrawing OM from the medium caused the cytoplasmic vesicles to slowly disappear. After 3 days of culture in the absence of OM, most of the cells were morphologically indistinguishable from control cells.

OM antagonizes EGF mitogenic activity. To study potential mechanisms of growth inhibition, we investigated whether OM inhibited the mitogenic activity of a variety of epithelial cell mitogens. Initial studies focused on EGF, as EGF has been shown to stimulate the growth of normal and malignant mammary epithelial cells, and reports from several groups have suggested that the EGF ligand/receptor system may play an important role in the proliferation of breast cancer cells and in tumor development. Treatment of H3922 for 3 days with EGF stimulated increased DNA synthesis with an EC_{50} of approximately 80 pg/ml (Figure 2A). Maximal increases of 2 to 4-fold in DNA synthesis were routinely obtained at concentrations of 1 ng/ml and higher. To examine whether OM can antagonize the proliferative activity of EGF H3922 cells were stimulated with 1 ng/ml of EGF in the presence or absence of OM. As shown in Figure 2A, OM blocked EGF induced DNA synthesis in a concentration-dependent manner. The increased proliferative activity with the addition of EGF was antagonized completely by OM at a concentration of 20 ng/ml and higher. The inhibition of EGF stimulated cellular proliferation by OM was also confirmed by cell number count as shown in Figure 2B. The effect of OM on the growth of H3922 cells was then examined under anchorage-independent conditions. EGF was found to be required for the growth of H3922 in soft agar. However, the addition of OM together with EGF totally prevented the growth of H3922 in soft agar.

OM does not affect EGF receptor expression or tyrosine kinase activity. To determine whether OM modulates EGF receptor expression or signaling, we examined the binding of [^{125}I]EGF and EGF-induced receptor tyrosine phosphorylation in control cells and OM treated cells. As shown in Figure 3A, H3922 cells showed a dose dependent binding of ^{125}I-EGF with an affinity of 1.1 nM and 2.97 x 10^6 sites/cell. Pretreatment of H3922 cells for 24 hours with OM did not decrease ^{125}I-EGF binding. Stimulation of H3922 cells with EGF induced rapid tyrosine phosphorylation of a protein of approximately 190 kDa. This phosphorylated protein was confirmed to be the EGF receptor by western blot analysis of the cell lysate obtained from EGF-treated cells using an anti-EGF receptor monoclonal antibody. EGF also induced phosphorylation of two other proteins with apparent molecular weights of approximately 55 kDa and 30 kDa. The EGF induced phosphorylation of the EGF receptor and the other two proteins was only slightly decreased in the cells that had been pretreated with OM for 2 days (Figure 3B). These studies shows no significant changes in EGF binding and signalling, and therefore suggest that the mitogenic effects of EGF may be blocked by OM at step(s) downstream from the EGF receptor.

The effects of OM on growth stimulation by other epithelial cell growth factors. A number of the breast cancer cell lines including H3396 and H3630 did not respond to EGF stimulation, but these cells were growth inhibited by OM. This suggests that the anti-EGF activity of OM may not be the only mechanism by which OM affects cell growth. To test this possibility, we initially utilized anti-EGF neutralizing monoclonal antibody to determine if OM would lose its inhibitory activity under conditions in which the activity of EGF present in the medium had been neutralized. Therefore, cell growth assays of H3922 were conducted in the presence or absence of 5 μg/ml of neutralizing anti-EGF mAb. OM was found to inhibit cell growth (~50% inhibition) even in the presence of added anti-EGF mAb. The concentration of anti-EGF mAb used inhibited > 80% of the increased cell growth stimulated by exogenously added EGF (Table I). These data suggest that OM growth inhibitory activities may include the antagonism of other growth factors which are present in serum. Therefore, we examined the ability of OM to inhibit the activities of several growth factors which have been shown to stimulate growth of normal or malignant epithelial cells. Insulin, IGF-1, and PDGF over a broad range of concentrations (from ng/ml to μg/ml) did not induce H3922 cells to proliferate. However, members of the EGF family of growth factors, AR, hbEGF, and TGF-α stimulated DNA synthesis in H3922 cells. Each of the growth factor induced a ~ 4-fold increase in cellular proliferation. The increased proliferation was blocked by 80-100% when OM was included in the culture medium with each of the growth factors (Figure 4).

It has been shown that bFGF stimulates the growth of cells derived from different breast tumors, and the levels of bFGF in the urine of patients with variety of cancers including breast tumors are elevated, suggesting a role of bFGF in breast cancer *in vivo*. We therefore examined the proliferation response of H3922 cells to bFGF in the presence or absence of OM. Figure 4 shows that bFGF is a mitogen for these cells increasing DNA synthesis by 3.5 fold at a concentration of 10 ng/ml. OM was able to completely block the increased proliferative activity of bFGF treated cell cultures.

The ability of OM to antagonize EGF and bFGF induced cell growth were further tested in another breast cancer cell line ZR-75-1. As shown in Figure 5, both EGF and bFGF increased DNA synthesis in ZR-75-1 cells in a similar fashion to the results seen with H3922 cells. OM was also able to antagonize the mitogen induced proliferation of both EGF (~50%) and bFGF (~80%). This result shows that the antagonism of growth factor dependent cell growth by OM is not limited in H3922 cells and may represent a general activity of this cytokine for breast cancer cells.

TGF-β is known to inhibit growth of normal and transformed epithelial cells. To determine whether the effect of OM on breast cancer cells is mediated by production of TGF-β, H3922 cells were treated with OM and TGF-β respectively, in the presence and the absence of TGF-β neutralizing antibodies. The TGF-β neutralizing antibodies totally prevented the TGF-β induced growth inhibition of H3922 cells, but had no effect on OM activity (Figure 6). Furthermore, these antibodies did not affect the cell growth cultured in normal medium. These results suggest that TGF-β is not involved in OM mediated growth inhibition of breast cancer cells.

Suppression of c-myc gene expression. We next examined the regulation of *c-myc* gene expression by OM, as this gene product plays a central role in the regulation of cell growth and differentiation. Much evidence links *c-myc* gene expression with cell proliferation, and reduction of its expression correlates with cellular differentiation. Further, over expression of the *c-myc* gene has been found in 15-40% of the breast tumors tested. H3922 cells were cultured in the presence or absence of OM for 3 days prior to stimulation with EGF for 4 hours. The mRNAs isolated from these cells were analyzed by northern blot for the presence of the *c-*

myc gene transcript as well as another cell growth-related gene, *c-jun*. As shown in Figure 7, the basal expression of *c-myc* in OM treated cells was reduced to approximately 15 to 20% of that observed in control cells. EGF stimulation increased *c-myc* mRNA level to approximately 180% of controls. This increased *c-myc* expression was completely abolished in EGF stimulated cells that had been pretreated with OM. In contrast to the changes seen in *c-myc* mRNA expression, the basal expression and the EGF-stimulated expression of *c-jun* was not suppressed at all in OM treated cells. In fact, the mRNA level of *c-jun* was increased in OM treated cells to approximately 166% of control. These data suggest that the *c-myc* gene transcription is regulated selectively by OM. The results clearly showed that OM not only suppresses the constitutive expression but also antagonizes mitogen stimulated expression of the *c-myc* gene.

Comparison of OM activity with related cytokines. The biological activities of OM can be mediated by two related receptors that bind OM. One receptor has been found to be OM-specific. A second, more promiscuous receptor has been shown also to bind LIF and more recently CT-1. In addition to OM sharing a receptor with related cytokines, many biological responses of OM are common to LIF, IL-6, and IL-11. These effects can be exerted by either the same receptor or related receptors each containing a common signal transduction unit, gp130. To determine which receptor complex is responsible for transmission of the OM anti-growth signal in breast cancer cells, we performed competition binding assays in H3922 cells. The result showed that [^{125}I]OM binding to H3922 cells was displaced by unlabeled OM, but not by unlabeled LIF (Figure 8). This suggested a direct binding to the OM-specific receptor. We therefore tested the growth regulatory activity of LIF, IL-6, and IL-11. The results are described in Table II. LIF, IL-6, and IL-11 did not inhibit DNA synthesis of the cells in the presence or the absence of exogenous EGF, suggesting that these cytokines do not repress proliferation of the malignant epithelial cells driven either by serum or by EGF. In fact, cell growth was slightly stimulated by these factors. Consistent with the different effect of these cytokines on cell growth, northern blot analysis showed that LIF, IL-6, and IL-11 did not significantly decrease the level of *c-myc* mRNA in H3922 cells (Figure 9). These results suggest that the OM growth inhibitory activity in breast cancer cells is a function of this cytokine that is not shared with related cytokines, and that the activity is mediated predominantly through the OM-specific receptor, not the shared LIF/OM/CT-1 receptor.

Effects of OM on normal human epithelial cells. To investigate whether OM has a similar effect in normal human mammary epithelium (HME), growth assays were conducted in normal primary HME cells derived from four donors. OM produced a dose-dependent inhibition of DNA synthesis in these cells (cultured in mammary epithelium growth medium) (Figure 10). In contrast, leukemia inhibitory factor (LIF), a cytokine closely related to OM, had no effect on HME cell proliferation (Figure 11). Flow cytometry analysis using antigp130mAb and antiOMRβmAbs (the monoclonal antibodies against the second subunit of OM specific receptor, provided by Immunex) demonstrated that these cells express both receptor subunits, but the OMRβ is expressed at higher level than found in breast cancer cells (Figure 12). We then examined the mRNA levels of each OM receptor subunit including gp130, OMRβ and LIFR in different breast cancer cell lines and in HME cells. The quantitative PCR analysis demonstrates that the mRNA level of OMRβ is much higher in HME cells compared with breast cancer cells. The LIF receptor messenger is expressed at a lower level in both normal and malignant mammary epithelial cells compared with the levels of OMRβ mRNA (Table III).

Conclusion

The results described in this report demonstrate that

1) OM has inhibitory and different activities in both normal and malignant mammary epithelial cells.

2) The inhibitory mechanism in breast cancer cells involve antagonism to a variety of breast cancer mitogenes and down regulation of c-myc protooncogene.

3) OM activities can not be mimiced by LIF and other related cytokines. That suggest the OM-specific receptor not the OM/LIF shared receptor transduces the OM signal in normal and malignant mammary epithelial cells.

4) Both normal and malignant mammary epithelial cells express the LIF receptor at a very low level compared with the expression level of OM-specific receptor.

5) The expression levels of the OM-specific receptors are decreased or lost in breast cancer cell lines.

Based upon these results we suggest that OM plays a physiological role in breast malignancy. The deregulation of OM-specific receptor renders the tumor cells to escape from the negative growth control which contributes to tumorigenesis.

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Figure 1

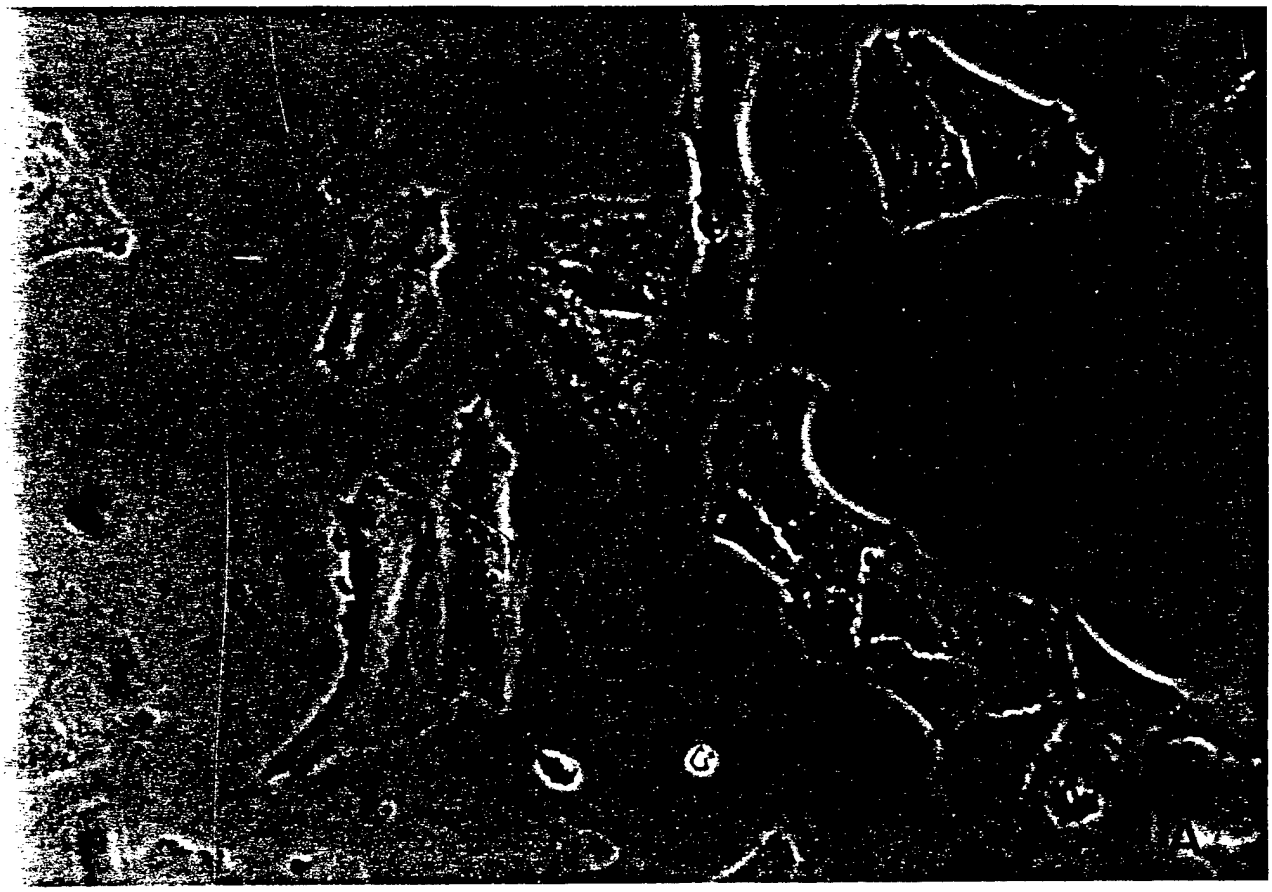


Figure 2A

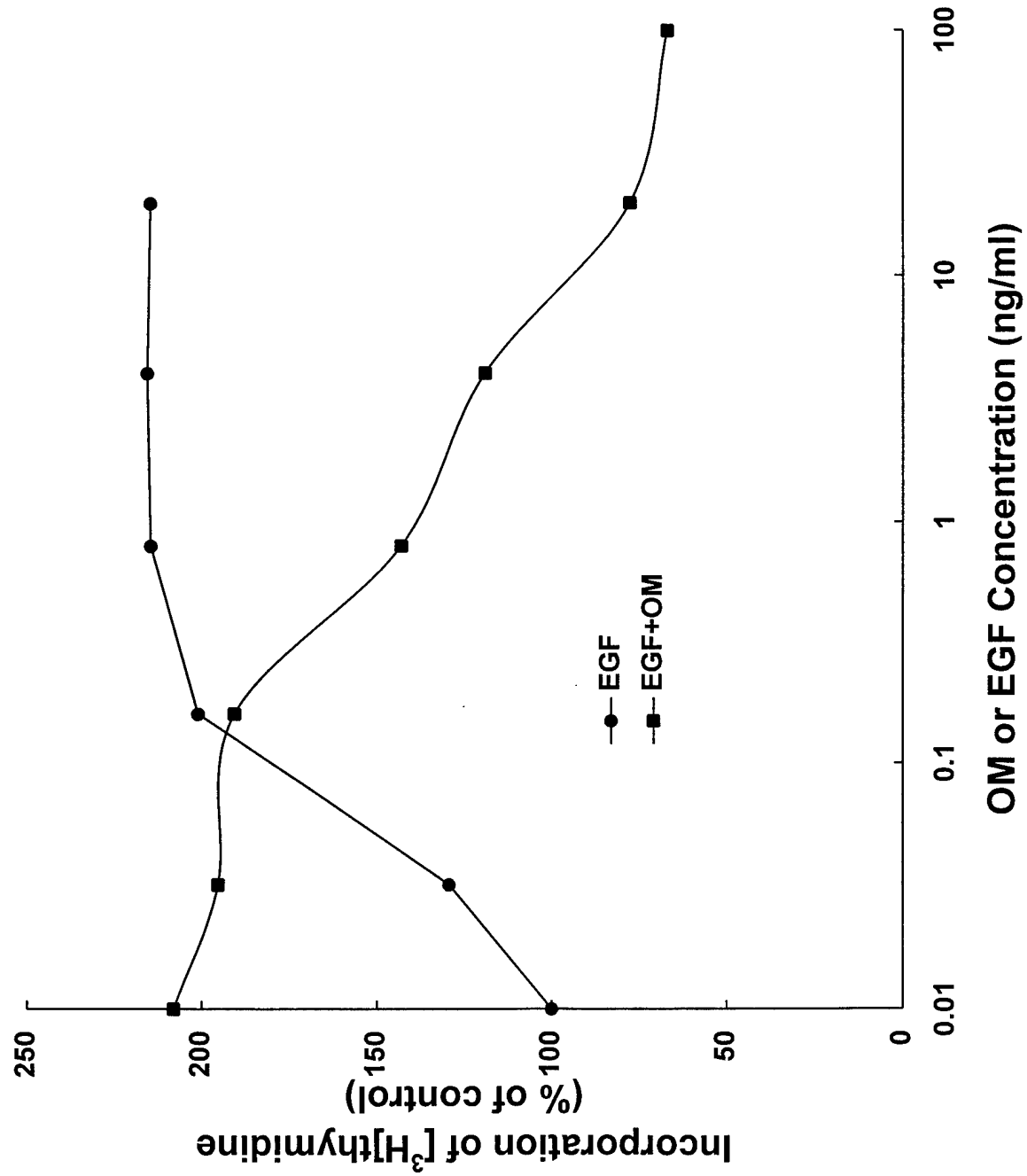

Incorporation of [3H]thymidine
(% of control)
250
200
150
100
50
0
0.01
0.1
1
10
100
OM or EGF Concentration (ng/ml)
EGF
EGF+OM

Figure 2B

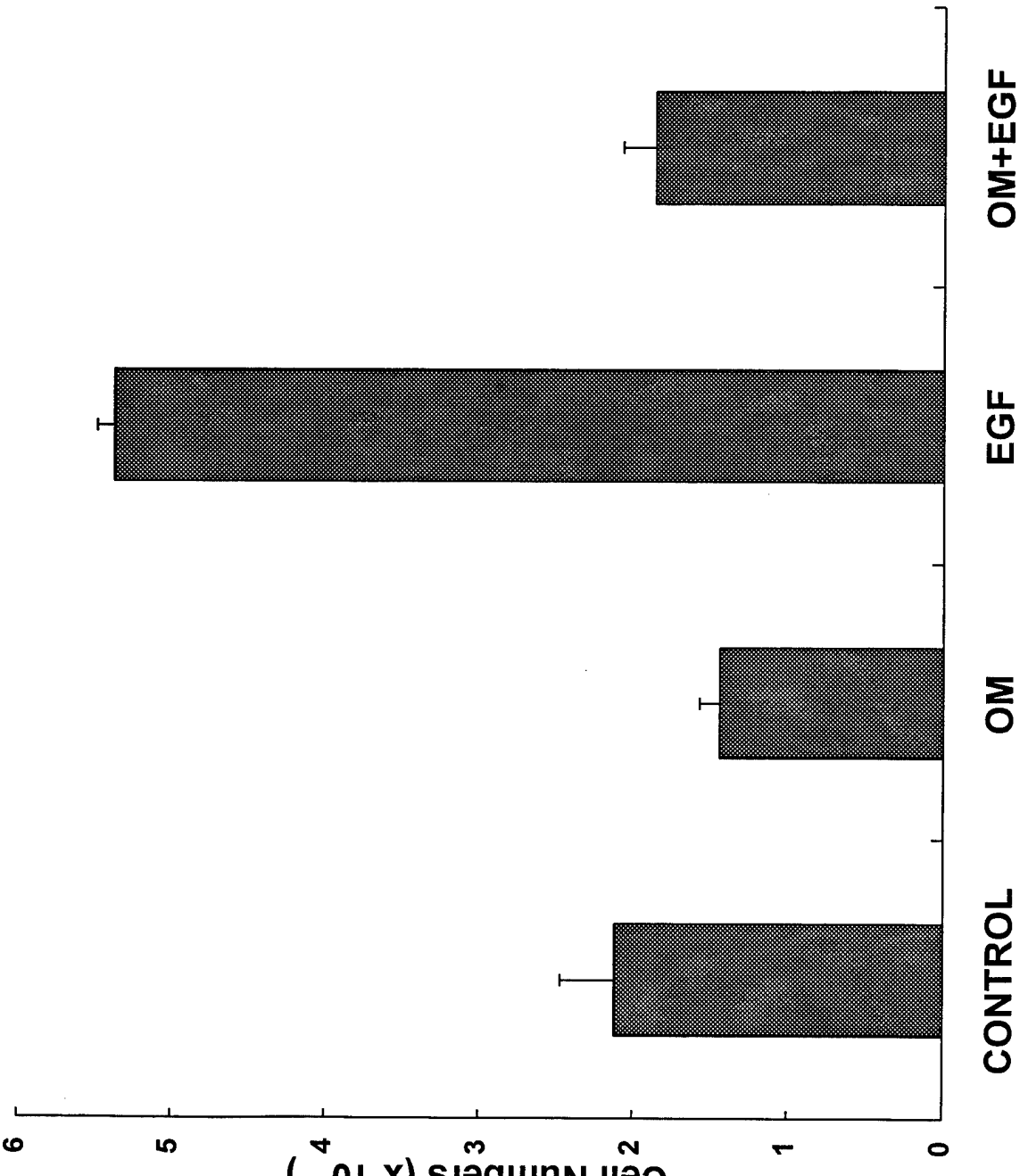


Figure 3A

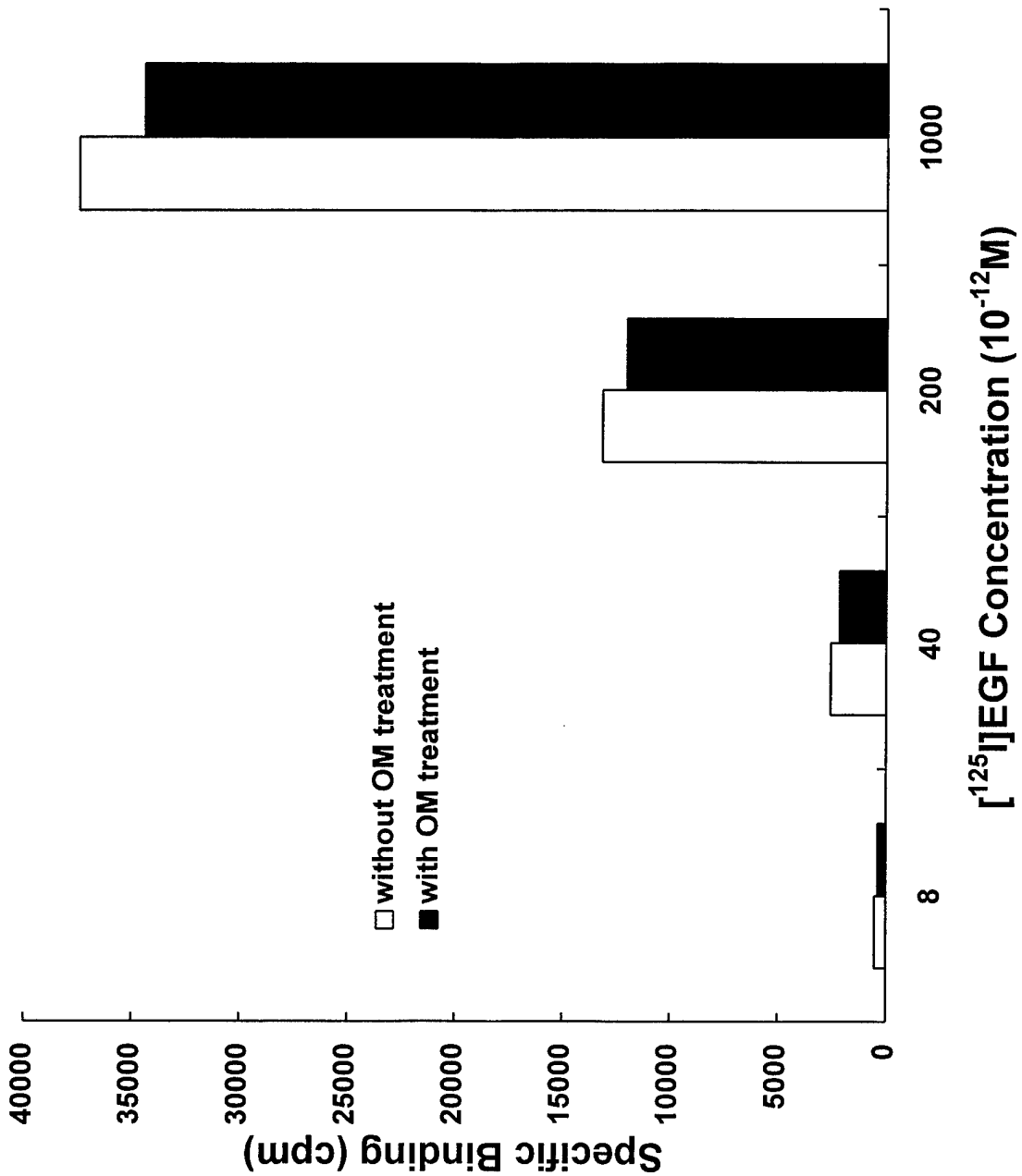

Specific Binding (cpm)
40000
35000
30000
25000
20000
15000
10000
5000
0
without OM treatment
with OM treatment
8
40
200
1000
[125I]EGF Concentration (10-12M)

Figure 3B

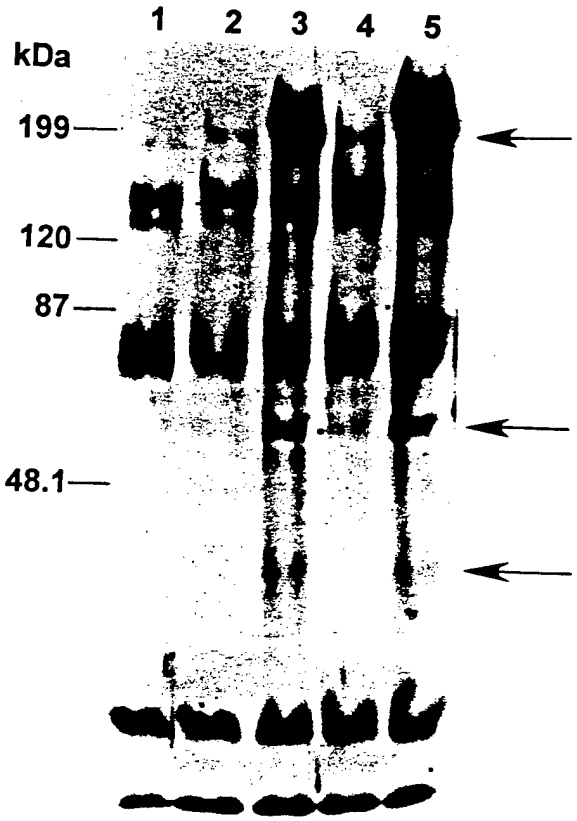


Figure 4

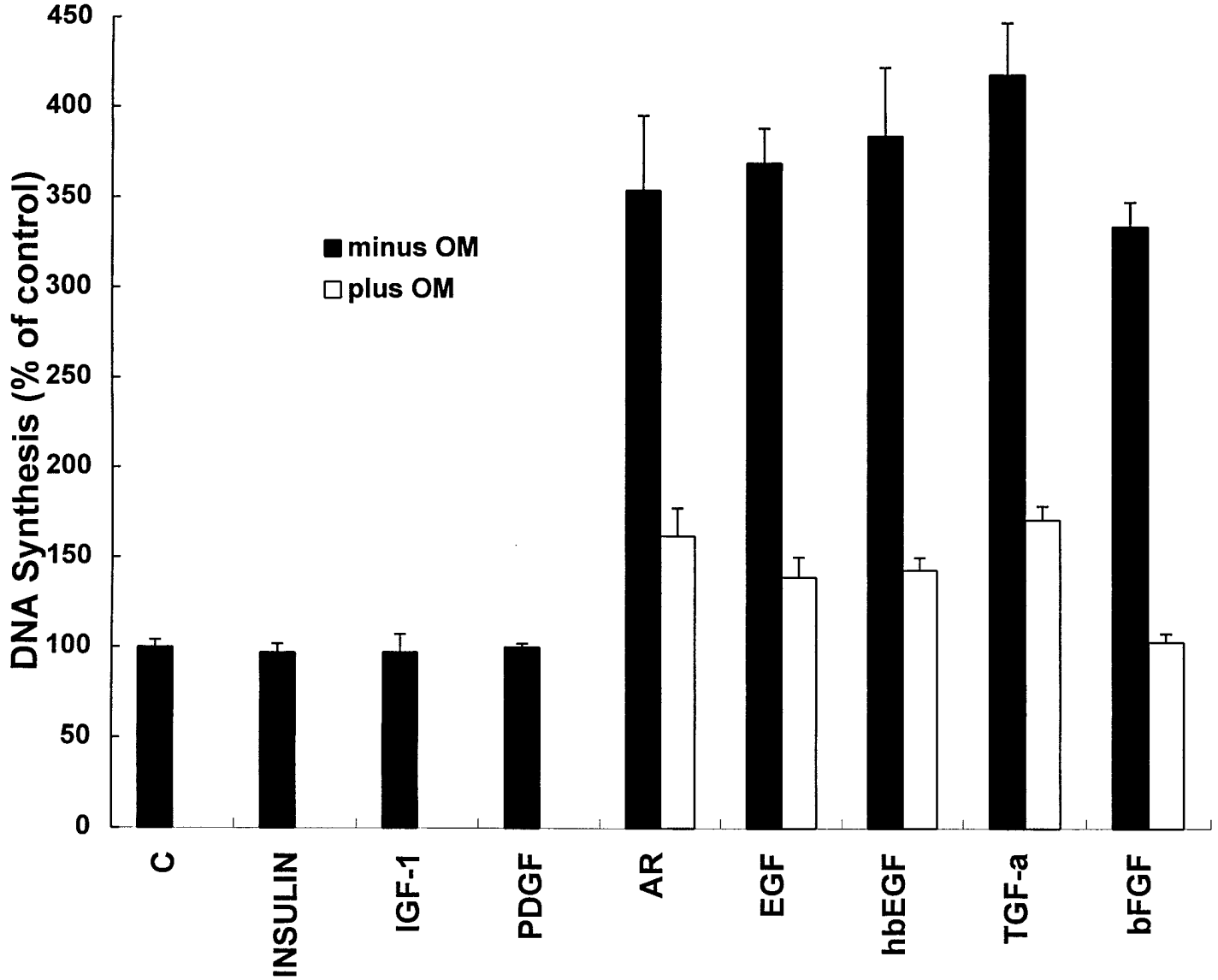

DNA Synthesis (% of control)
450
400
350
300
250
200
150
100
50
0
minus OM
plus OM
C
INSULIN
IGF-1
PDGF
AR
EGF
hbEGF
TGF-a
bFGF

Figure 5

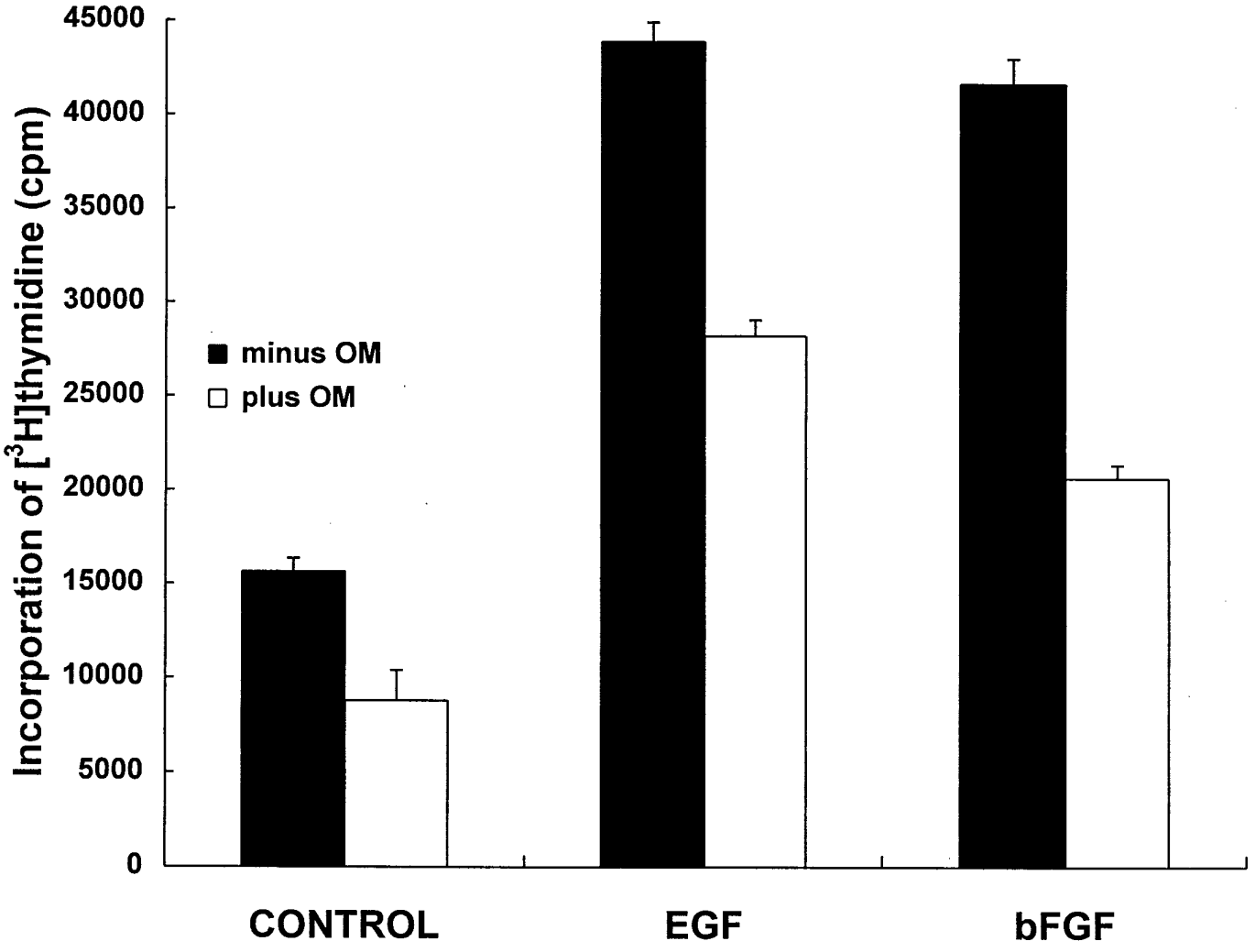

Incorporation of [^{3}H]thymidine (cpm)
45000
40000
35000
30000
25000
20000
15000
10000
5000
0
minus OM
plus OM
CONTROL
EGF
bFGF

Figure 6

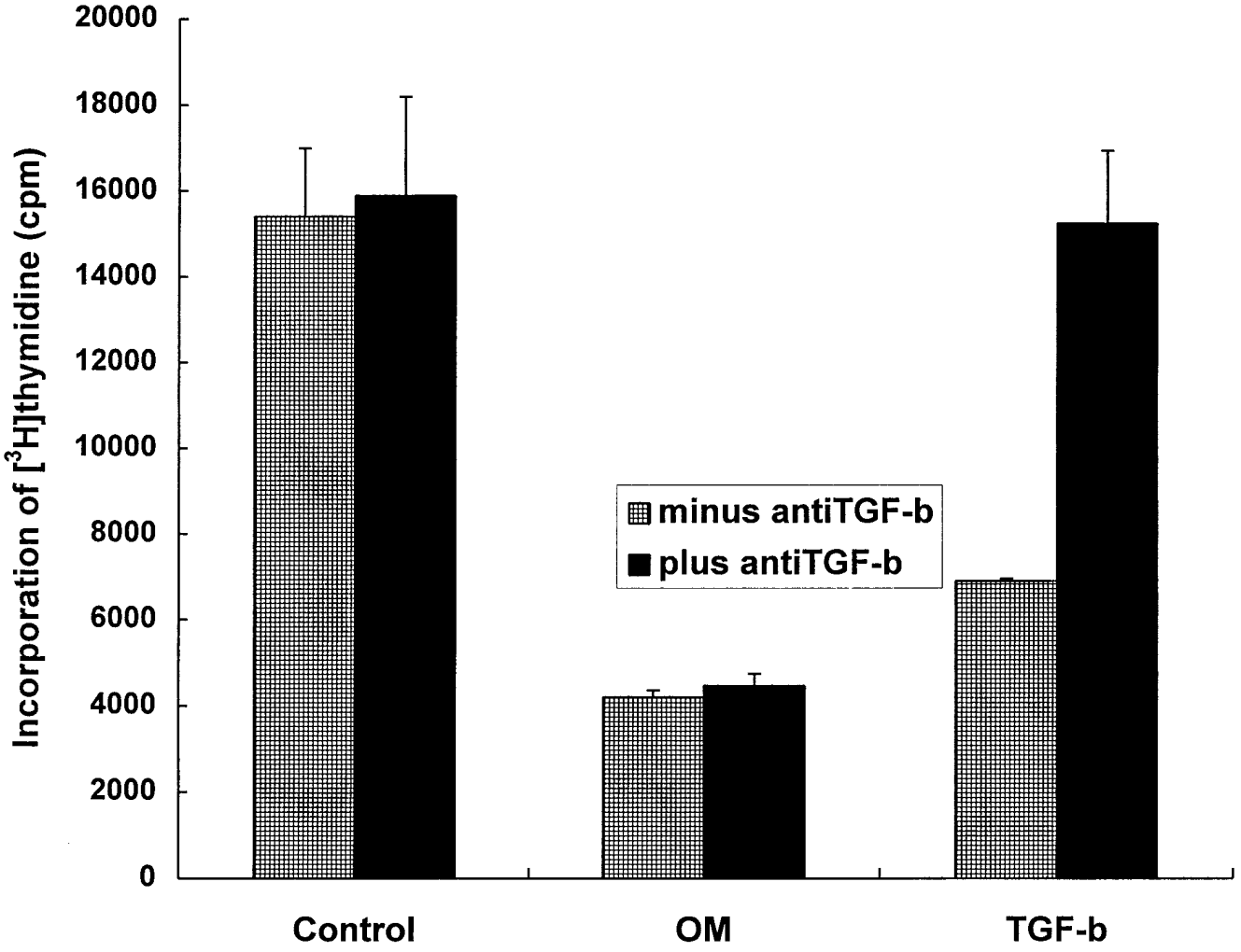
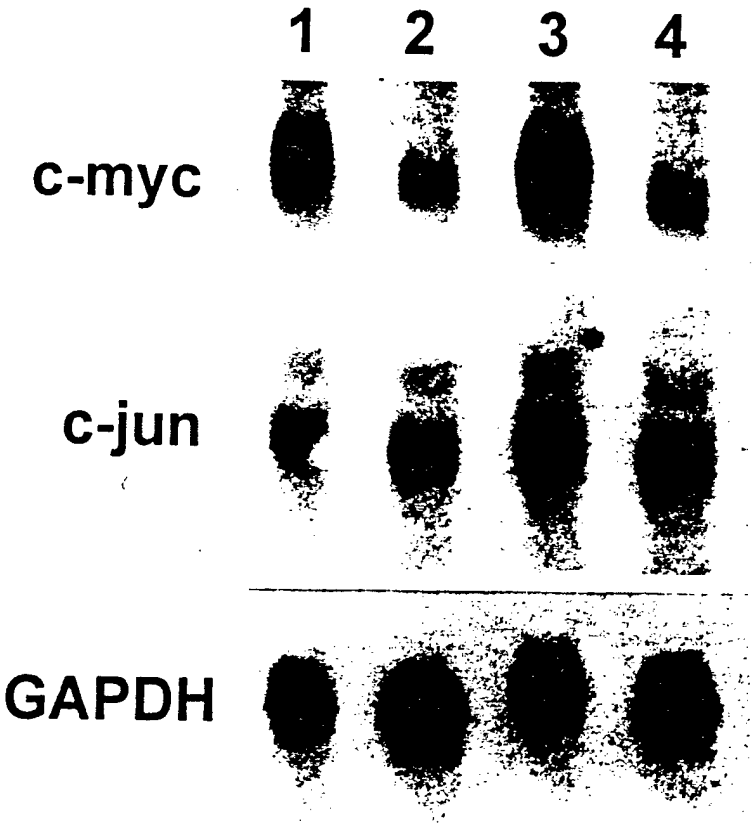

20000
18000
16000
14000
12000
10000
8000
6000
4000
2000
0
Incorporation of [3H]thymidine (cpm)
minus antiTGF-b
plus antiTGF-b
Control
OM
TGF-b

Figure 7



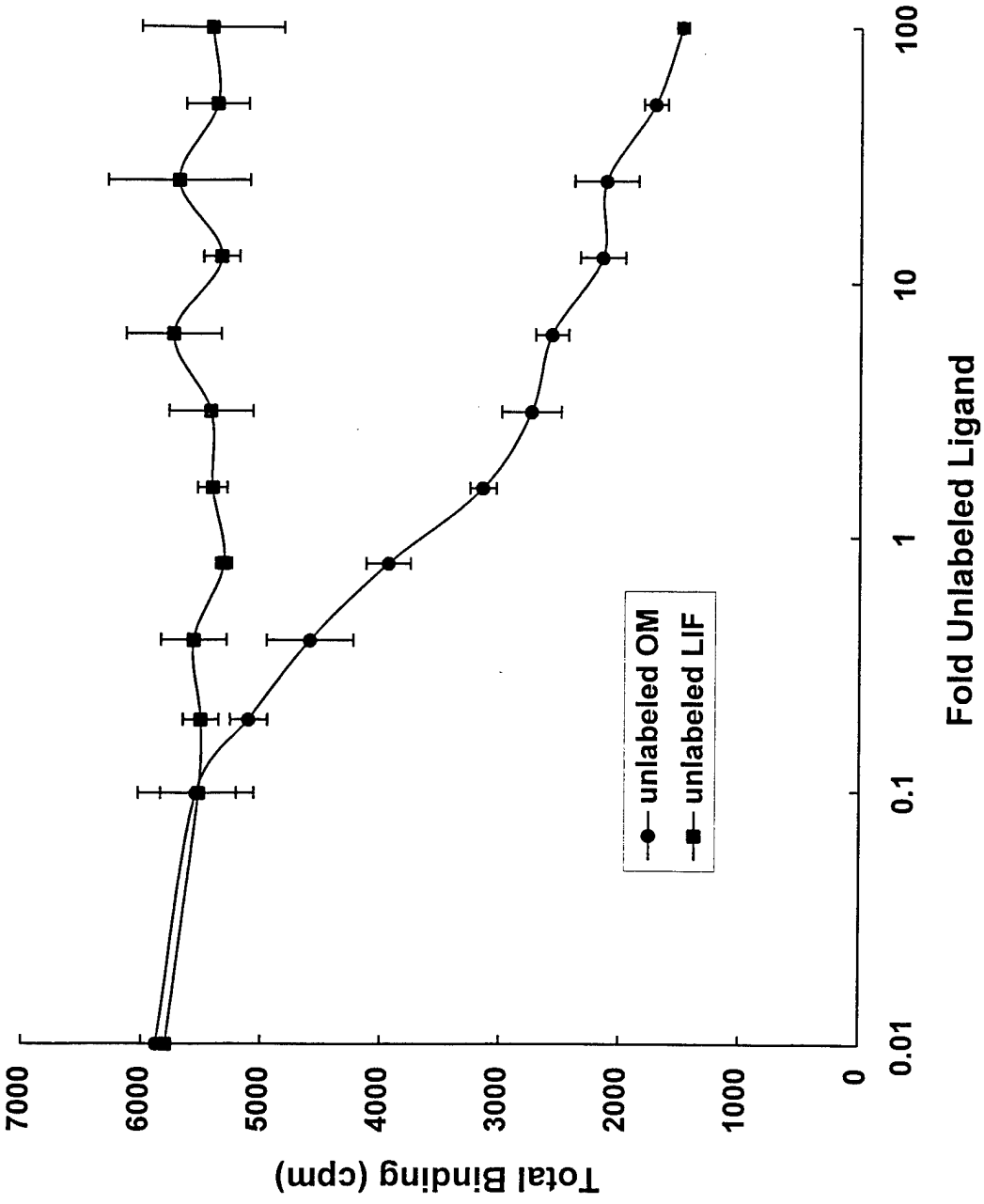

Total Binding (cpm)
7000
6000
5000
4000
3000
2000
1000
0
0.01
0.1
1
10
100
Fold Unlabeled Ligand
unlabeled OM
unlabeled LIF

Figure 8

Figure 9

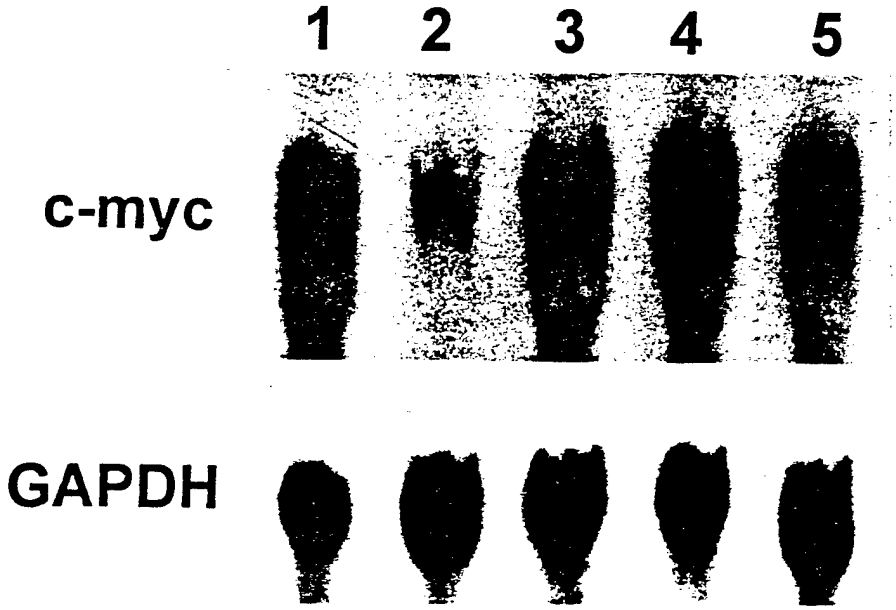
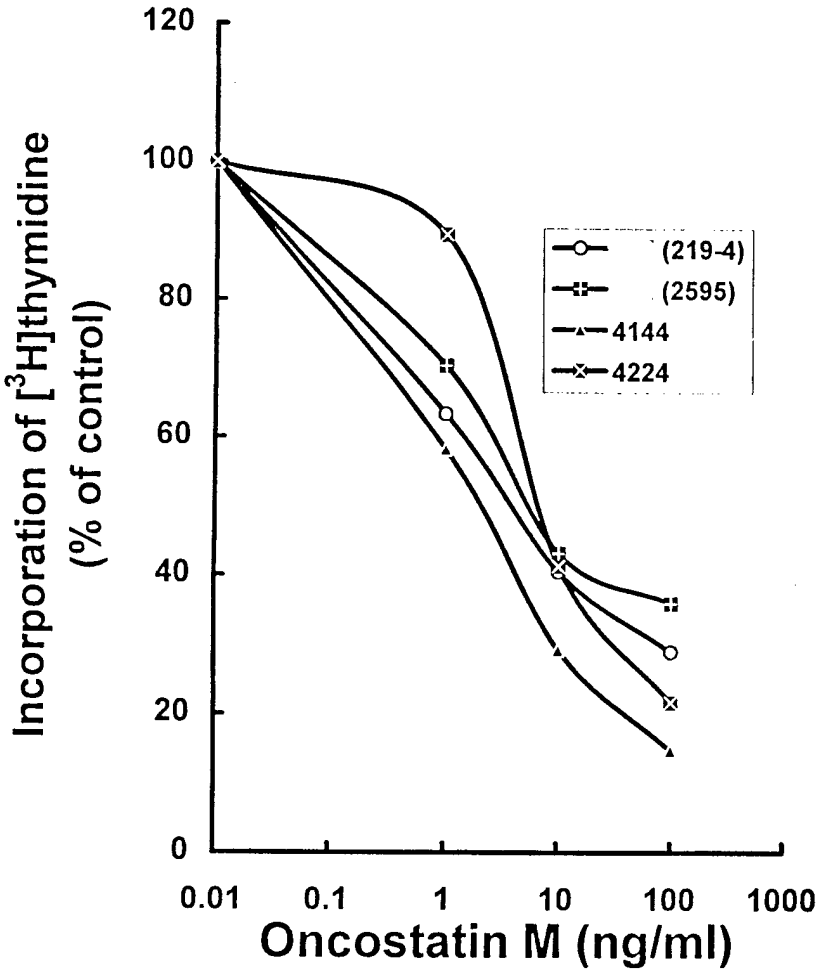


Figure 10

Oncostatin M inhibits the growth of normal HME cells



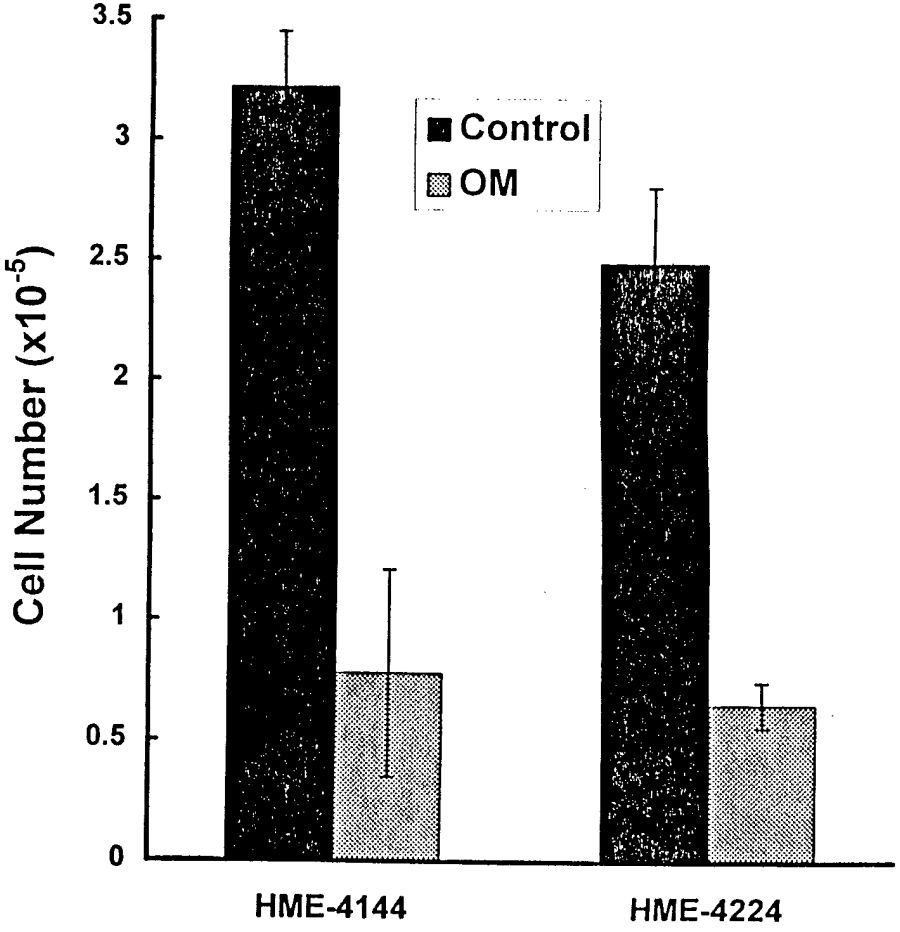


Figure 11

Comparison of the growth effects of OM and LIF on HME cells

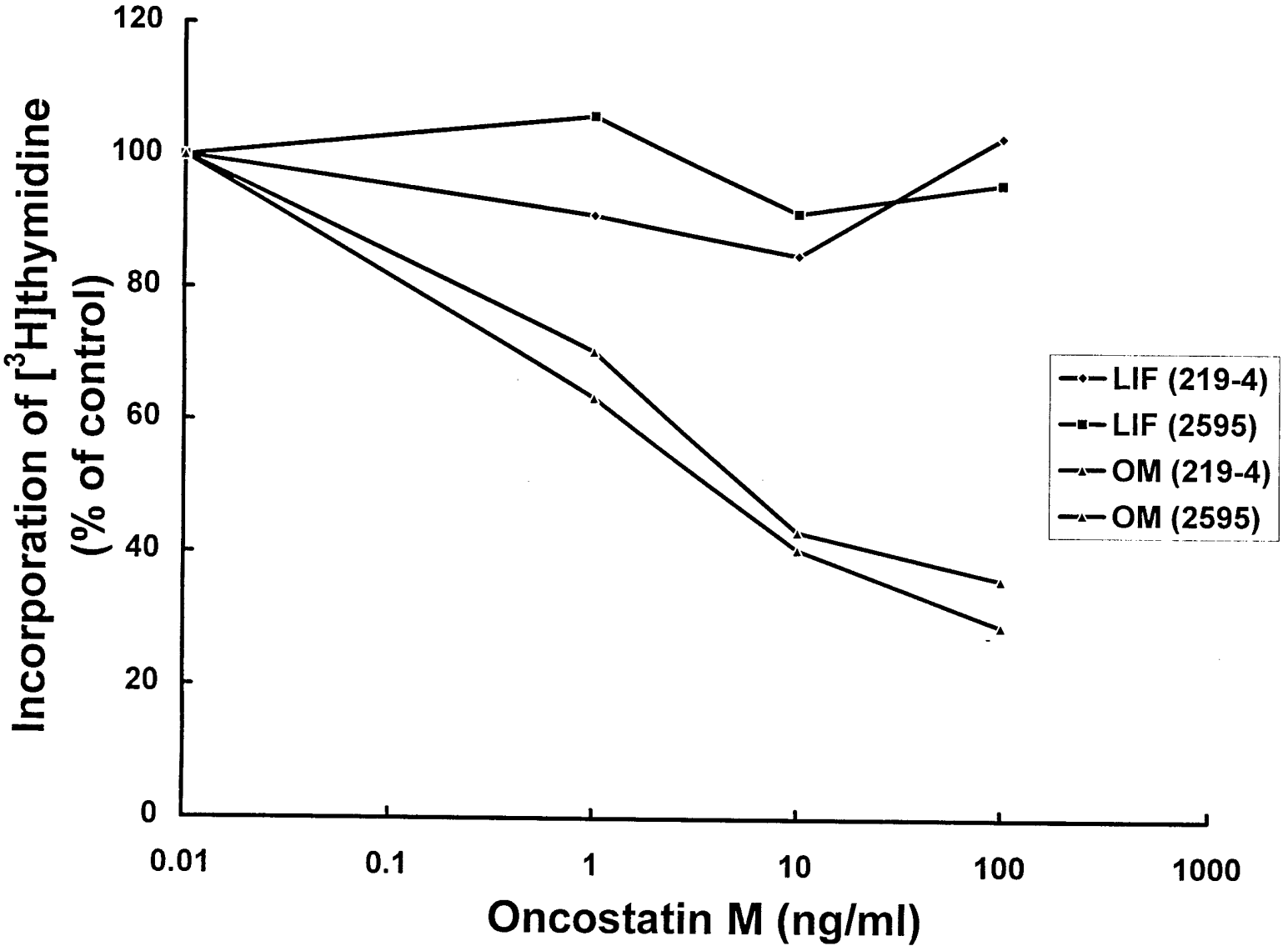


Figure 12

	H3922		HMEC		H3477	
	% Cell	Media	% Cell	Median	% Cell	Median
IgG	4.9	371.8	5.5	40.6	5.3	7.23
gp130	76.2	1064.9	8.0	42.5	40.63	22.6
OSMRβ	76.2	1055.5	68.0	181.1	18.9	10.1

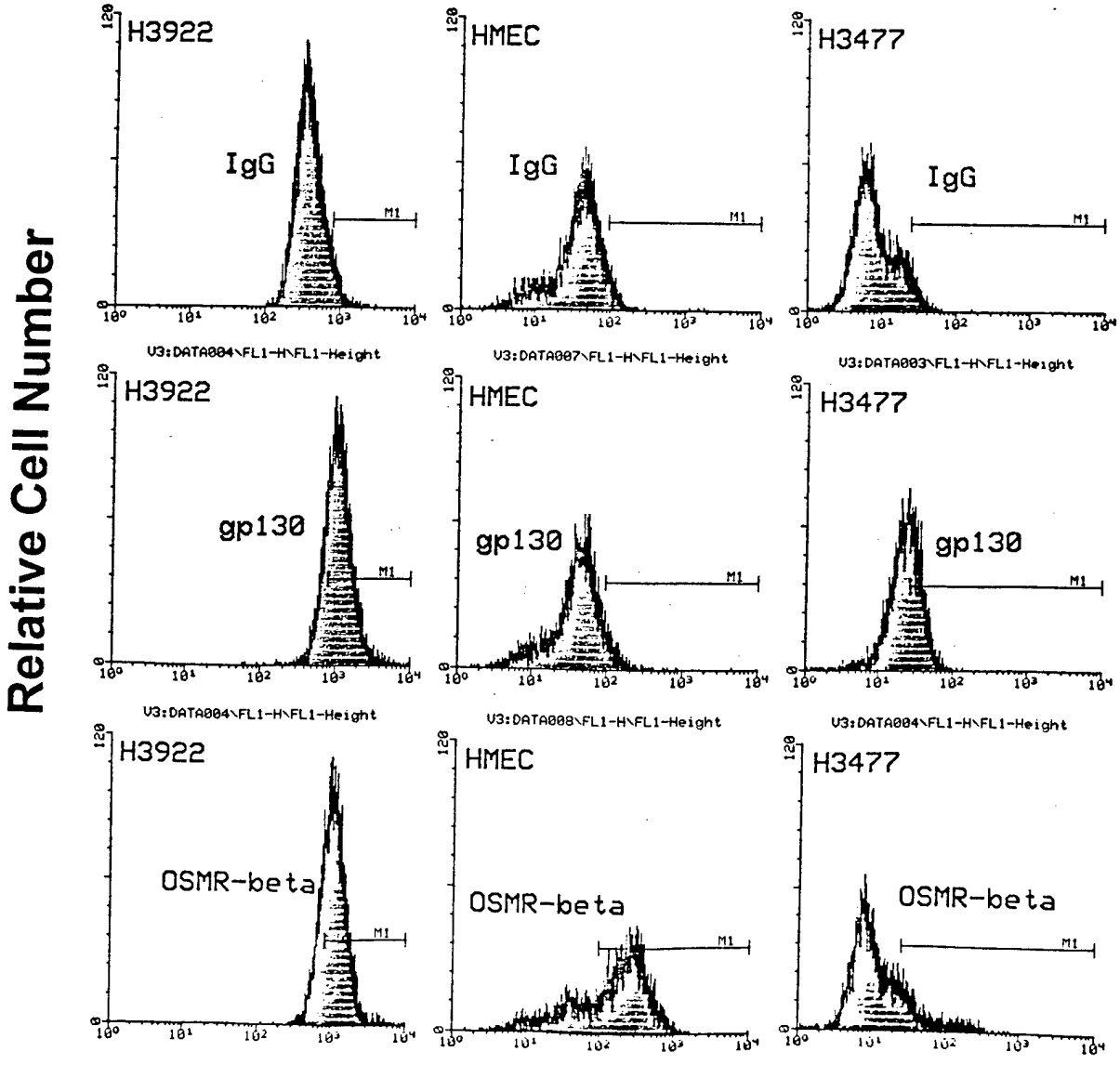


Figure Legends

Figure 1. Morphological change of H3922 breast cancer cells induced by OM. Cells were cultured in 6-well culture plates at a density of 1 x 10^5 cells/well in 2.5 ml 10% FBS IMDM supplemented with OM. Photographs were taken after 3 days of initial culturing (200 x magnification). A, medium alone; B, OM, 20 ng/ml.

Figure 2. OM antagonized EGF proliferative activity. (A) H3922 cells (1 x 10^3 cells/well) were incubated for 3 days in IMDM containing 10% FBS supplemented with various amounts of purified human recombinant EGF, or with 1 ng/ml EGF plus the indicated amount of OM. Cells were pulsed with [^{3}H]thymidine for an additional 4 hours. The amount of radioactivity incorporated into cells was determined, and the data expressed as the percentage of radioactivity compared to cells that were not treated with factors. (B) H3922 cells were cultured in 6-well culture plates at a density of 1 x 10^5 cells/well in 2.5 ml 10% FBS IMDM supplemented with 20 ng/ml OM, 10 ng/ml EGF, or 20 ng/ml OM plus 10 ng/ml EGF respectively. Three days later, cells were trypsinzed and trypan blue excluding cells were counted (>3 independent assays). Values are mean ± SD.

Figure 3. EGF binding and tyrosine kinase activity of EGF receptor were not affected by OM treatment. (A) H3922 cells cultured in 48-well culture plates were treated with 100 ng/ml of OM or OM diluent (PBS) for 24 hours. The cells were washed with cold PBS (4° C) and then the binding of [^{125}I]EGF at the indicated concentration was determined. (B) H3922 cells were cultured in 2% FBS IMDM with or without 100 ng/ml OM for 2 days. Then the cells were stimulated with 10 ng/ml of EGF for 10 minutes, or 100 ng/ml of OM for 15 minutes. The immunoprecipitation and detection of tyrosine phosphorylation were conducted as described in Methods. Lane 1, control; lane 2, OM for 2 days; lane 3, OM for 2 days then stimulated with EGF for 10 minutes; lane 4, 15 minutes of OM alone, and lane 5, EGF for 10 minutes without OM pretreatment.

Figure 4. Inhibitory activity of OM on other growth factors. H3922 cells were incubated for 3 days in 10% FBS IMDM supplemented with different growth factors in the presence or absence of 10 ng/ml of OM. The concentrations of growth factors used were EGF, TGF-α, and bFGF, 10 ng/ml; amphiregulin, 1 μg/ml; hbEGF, IGF-1, and PDGF, 100 ng/ml; insulin, 5 μg/ml. Bars are mean ± SD. The dose dependent effects of these factors have also been examined and the results were consistent with the data presented.

Figure 5. Anti-EGF and anti-bFGF proliferative activities of OM on ZR-75-1 cells. ZR-75-1 cells (1 x 10^3 cells/well) were incubated for 3 days in 2% FBS IMDM supplemented with EGF (100 ng/ml), or bFGF (50 ng/ml) in the presence or absence of 20 ng/ml of OM. Growth effects were determined as in Figure 1.

Figure 6. OM inhibitory activity can not be abrogated by Anti-TGF-β neutralizing mAb. H3922 cells were cultured for 3 days in 10% FBS IMDM supplemented with OM (50 ng/ml), or TGF-β (2 ng/ml) in the presence or absence of 5 μg/ml anti-TGF-β neutralizing mAb. The cell proliferation was measured as described in Figure 1.

Figure 7. Down regulation of the *c-myc* mRNA by OM in breast cancer cells. H3922 cells were cultured in the presence or absence of OM for 3 days. Then the cells were stimulated with EGF (10 ng/ml) for 4 hours before harvesting. Total RNAs were isolated from these cells and 20 μg/lane was analyzed by northern blot for the presence of the *c-myc* gene and *c-jun* gene transcript. The RNA blot was stripped and reprobed for *GADPH* subsequently to ensure the equal loading. The intensity of hybridization signal was quantitated by a laser densitometer (Molecular Dynamics, Sunnyvale, CA). lane 1, control; lane 2, OM for 3 days; lane 3, EGF for 4 h; lane 4, OM for 3 days plus EGF for 4 h.

Figure 8. Inability of LIF to displace [^{125}I]OM bound to H3922 cells. Cells were incubated with 2 nM of [^{125}I]OM in the presence of increasing concentrations of unlabeled OM, or unlabeled LIF. The total binding was determined as described in Table I.

Figure 9. Comparison of effects of OM related cytokines on *c-myc* gene transcription. Cells were incubated in 2% FBS IMDM containing the indicated cytokines (100 ng/ml) for 2 days, then stimulated with EGF (10 ng/ml) for 4 hours before harvesting. Total RNAs (20 μg/lane) were analyzed for *c-myc* mRNA by northern blot. Lane 1, EGF alone; Lane 2, EGF plus OM; Lane 3, EGF plus IL-6; Lane 4, EGF plus IL-11; Lane 5, EGF plus LIF.

Figure 10. Effect of OM on proliferation of HME cells.
(A) DNA synthesis. HME cells (1.2 x 10^4 cells/well) were incubated for 3 days in MEGM with indicated amount of purified human recombinant OM. Cells were then pulsed with [^{3}H]thymidine for 4 hours. The amount of radioactivity incorporated into cells was determined by TCA precipitation method, and the data are expressed as the percentage of radioactivity incorporated compared to untreated cells. (B) Cell number count. HME cells were cultured in 4-well culture plates at a density of 1.7x10^5 cells/well in 5 ml MEGM supplemented with 100 ng/ml of OM. Three days later, cells were trypsinzed and trypan blue excluding cells were counted. Values are mean $\pm$SD.

Figure 11. Comparison of growth inhibitory effect of OM and LIF. HME cells (1.2 x 10^4 cells/well) were incubated for 3 days in MEGM with indicated amount of purified human recombinant OM or LIF. Cells were then pulsed with [^{3}H]thymidine for 4 hours. The amount of radioactivity incorporated into cells was determined by the TCA precipitation method, and the data are expressed as the percentage of radioactivity incorporated compared to untreated cells.

Figure 12. Comparison of the expression of OM receptor subunits protein on HME and breast cancer cells detected by immunofluorescence. Cells (1 x 10^6 cells/ml) were incubated with 10 μg/ml of anti-gp130mAb, anti-OMRβmAbs, or $IgG2_a$ then stained with FITC-conjugated anti-mouse antibodies and analyzed by flow cytometry. % Cell values indicate percentage of cells stained. Median values indicate median fluorescence intensity.

Table I. Effects of neutralizing anti-EGFmAb on H3922 cell proliferation

	Addition	DNA synthesis (cpm) - Anti-EGFmAb	DNA synthesis (cpm) + Anti-EGFmAb
Assay #1	10% serum	6,733 ± 468	4,692 ± 316
	+ OM	2,138 ± 147	2,309 ± 167
	+ EGF	30,696 ± 1,733	6,948 ± 240
Assay #2	10% serum	9,820 ± 791	8,890 ± 216
	+ OM	5,591 ± 322	5,058 ± 117
	+ EGF	17,740 ± 700	10,602 ± 1,201

The growth assays were conducted with H3922 cells cultured in control medium (10% serum), or supplemented with 20 ng/ml of OM, or 10 ng/ml of EGF in the presence or absence of 5 μg/ml of anti-human EGF neutralizing mAb. Values are mean ± SD of triplicate cultures.

Table II. Specificity of the anti-EGF activity of OM on breast cancer cells

Cytokine	Cytokine Concentration (ng/ml)	DNA synthesis (% of control) - EGF	+ EGF
None		100	425
OM	100	56	151
LIF	100	114	380
IL-6	100	146	390
IL-11	100	130	419

Cells (1 x 10^3/well) were plated in 96-well plates in 10% FBS and IMDM. Three hours later the indicated concentrations of cytokines were added with or without EGF (10 ng/ml). After 3 days, the cells were incubated with [^{3}H]thymidine and incorporation of radioactivity into cells was determined as in Figure 1.

Table III

Analysis of OM receptor subunits mRNA by Quantitative PCR

Template	OMRβ	gp130	LIFR	DHFR	OM activity
H3922	639	471	68	500	Inhibition
H3630	186	542	3	500	Inhibition
H3396	205	246	37	500	Inhibition
H3477	---	280	---	500	No Response
H3914	---	---	---	500	No Response
HME	1154	1248	194	500	Inhibition
HME + OM	1711	990	81	500	Inhibition

A first strand cDNA synthesized from total RNA isolated from each cell line was used as a template for PCR amplification.
Data shown are thousands of pixels normalized for template efficiency. DHFR values indicate relative signal strength.

H. Publications (5/1995-4/1996)

1. Liu, J., Spence, MJ., Wallace, WP., Forcier, K., HellstrÖm, H., and Vestal, RE. Oncostatin M-specific receptor mediates inhibition of breast cancer cell growth, antagonism of growth factors, and down regulation of *c-myc* protooncogene. Submitted to Journal of Clinical Investigation (4/96)

2. Spence, MJ., Vestal, RE., and Liu, J. Suppression of c-myc gene transcription by Oncostatin M in breast cancer cells. Manuscript in preparation.

3. Liu, J., Hadjokas, N., Mosley, B., Vestal, R.E. Oncostatin M is a growth inhibitor for normal human mammary epithelial cells. Abstract, AACR, 1996.

4. Liu, J., Spence, MJ., Vestal, RE. Oncostatin M, A Growth Inhibitor Of Breast Cancer Cells: Activity And Mechanism.
Abstract, 18th annual San Antonio Breast Cancer Symposium, 1995.

5. Liu, J., Wallace, P.M., Vestal, R.E. Oncostatin M inhibits breast cancer cell growth. Abstract, AACR 1995.